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The Cultivation of Viruses of the Tick-Borne Encephalitis Group on Monkey-Heart Cells.

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The cytopathogenic effect of the tick-borne encephalitis virus in human embryonic skin-muscle tissue cultures (1) and the cytopathogenic effect of the louping ill virus in HeLa-cell cultures (2) have been described in recent years.

The data concerning the cytopathogenic effect of viruses are of great importance for the development of diagnostic methods for these diseases. The essential disadvantage of the cultures of human embryonic skin-muscle tissue, however, is the heterogeneity of the cellular material, which results in a failure to produce a standard material with a known sensitivity to a certain virus in the laboratory. The HeLa cells are free of this disadvantage, but their origin from a malignant tumor will not permit their use for the preparation of vaccines or diagnosticums for humans. Therefore, a study of the viral sensitivity of the strain of cells isolated by Salk from the heart of a cynomologus monkey (3) is important, both for the purpose of diagnosis and, possibly, also for the production of antiviral preparations.

The data in the literature indicating that some strains of normal cells acquire signs of malignancy in prolonged cultivation do not exclude their use for diagnosis.

In the present work, which was proposed and supervised by Ye. N. Levkovich, the sensitivity of monkey-heart cells to some strains of viruses of the tick-borne encephalitis group were studied.

Material and Methods. Five strains of the tick-borne encephalitis virus, three strains of the diphasic meningoencephalitis virus and two strains of the louping ill virus were utilized in the work. A short description of the strains are presented in table 1.

The monkey-heart cells were grown in matrasses (distilling flasks) containing nutrient medium No. 199 with 10%-20% calf serum. The change of the

nutrient medium was made within 5-6 days after the arrangement of the culture. On the 8th-9th day, when a cellular monolayer was being formed, the nutrient medium was poured off and a 0.25 % trypsin solution, prepared on Hank's solution, was added. After a 20-30 minute contact at 37°C the cells were separated from the surface of the glass, pipetted for the destruction of cellular conglomerates, centrifuged at 400 rpm for 10 minutes, and washed twice in Hank's solution by means of centrifugation. Then the cellular precipitate was diluted with nutrient medium in such a manner that one milliliter of the cellular suspension contained 160,000-200,000 cells. Into each test tube we introduced 0.25 ml of the cellular suspension and added one milliliter of nutrient medium. The test tubes were incubated at 37°C in horizontal racks. Inoculation of the tissue cultures was made on the 4th-5th day. As material for the inoculation we used 10-% brain suspensions and cultural fluids. We injected 0.1 ml of the virus-containing inoculant into each test tube and after 20 minutes of contact with the cells we added 0.9 ml of nutrient medium No. 199 containing 10 % calf serum. The passages were conducted after 4-5 days. The tissue cultures were inspected microscopically every day for 10 days. The presence of the virus was checked in each passage by means of intracerebral inoculation of white mice.

Results. All 10 strains of the viruses in the experiment propagated readily in the cultures of the monkey-heart cells. The viruses were passed through 5-8 cultural passages.

The dilution of the original inoculant reached 10^5 by the fifth passage, whereas the titers of the viruses in the same passage remained near the original ones that were received in the biological experiments on white mice. Particularly high titers were noted in the strains "Sof'in," I-40, and "Abesettarov." The cumulative titers of the strains "Sof'in" and "P-n" amounted to $10^{25.2}$ and $10^{29.8}$, respectively by the fifth passage. For the purpose of establishing the pattern of the viral build-up according to the days from the inoculation, we titrated the cultural fluids of the fifth passage on white mice.

As is evident from table 2 the maximum release of the virus into the cultural fluid was observed on the 2nd-6th day after inoculation. The presented data attest to the intense propagation of the viruses in the monkey-heart cells. Beginning with the first cultural passage all of the viral strains registered a marked cytopathogenic effect. After 24 hours of cultivation of the virus the cells had already begun to degenerate. On the 2nd-3rd day, half of the cells in the inoculated tissue cultures were destroyed from the effect of the virus's propagation. Total destruction of the cells was noted by the fifth day (Figs 1 and 2).

Thus, the maximum release of viruses into the cultural fluid coincided in time with the most intensive disintegration of the cells. The cytopathogenic effect of the strains "Abesettarov," 256, and "Volkhov-2," weakly expressed in the first two passages, became more intensive according to the degree of further subinoculation. The cytopathogenic effect was observed in all 5-8 passages conducted. Cellular destruction was unobserved in the control tissue cultures, to which a 10-% brain suspension from healthy mice had been added. The cytopathogenic effect of the viruses was also reproduced with a cultural fluid that was first kept 48 hours at a temperature of -20°C. With this, the time of the cytopathogenic effect's occurrence and its intensity were unchanged.

In order to determine the specificity of the cytopathogenic effect we inoculated tissue cultures with a mixture of immune guinea-pig serum, with a neutralization index above 10,000, in a 1:2 dilution with a 10-% brain suspension, or with a cultural fluid containing the viruses. No cytopathogenic effect was developed in any of the experiments in the tissue cultures inoculated by the mixture of the virus and the immune serum (fig 3). The phenomenon of extinction of the viruses' cytopathogenic effect by the immune serum was reproduced 2-3 times in the cultivation period with all of the strains under investigation.

Parallel titrations of the viruses were conducted in tissue cultures and on mice in order to explore the possibility of titration of the viruses in the tissue cultures according to cytopathogenic effect. Tissue cultures and white mice were inoculated with cultural fluids or brain suspensions of the virus in ten-fold dilutions. The tissue cultures were observed for a period of 10 days after inoculation, the white mice for a period of 20 days. The data from the experiments are presented in table 3. The titers of the cultural viruses in the tissue culture and in the mice were approximately the same. The difference in the titers did not exceed 0.5-1.0 of a logarithm sign. Brain suspensions of the strains "Sof'in" and "MO" were titrated in a tissue culture and in mice, just as the cultural viruses were. With this, the titer of the virus in lg $TTsPD_{50}$ was less than that of the lg LD_{50} by 1.5-2.0. Evidently this is because the virus was not adapted to the tissue culture.

The fact that we have established here, concerning the propagation and the regular cytopathogenic effect of the viruses of the tick-borne encephalitis group in monkey-heart cells, allows one to use this method for the titration of viruses as well as for the retention of strains. The presence of the viruses' cytopathogenic effect in cultures of a stable strain of monkey-heart cells creates the possibility for the development of a simple method of diagnosis for tick-borne encephalitis that would be available in the practical laboratory.

Conclusions

1. The viruses of tick-borne encephalitis, diphasic meningoencephalitis, and louping ill propagate actively in cultures of monkey-heart cells.

2. All of the investigated strains of the viruses induce a marked cytopathogenic effect from the first cultural passage. The cytopathogenic effect is retained in further subinoculation.

3. The specificity of the cytopathogenic effect of the viruses of the tick-borne encephalitis group is determined in the neutralization test with a homologous immune serum in a tissue culture.

4. A comparative titration of the viruses in a tissue culture and in the tests of intracerebral inoculation of white mice gives practically identical results.

Footnote

* - The cumulative titer represents the sum of the virus's titers in the cultural fluids of five consecutive passages.

Literature

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3. Salk, J.: Am. J. Pub. Health, 1957, v 47, p 1

Illustrations

Fig. 1. Cytopathogenic effect of the tick-borne encephalitis virus, strain "Sof'in;" fifth day after inoculation.

Fig. 2. Cytopathogenic effect of the louping ill virus, strain I-40; fifth day after inoculation.

Fig. 3. Cultures of monkey-heart cells that have been inoculated with a mixture of the tick-borne encephalitis virus (strain "Sof'in") and specific immune serum; normal cells.

Table 1

The characteristics of the strains of the tick-borne encephalitis group of viruses.

Virus	Strain	Region of isolation	Year of isolation	Material from which the virus was isolated	Titer of virus
Tick-borne encephalitis	"Sof'in	Far East	1937	Brain of deceased human	9.2
Same	"Khabarovsk-17"	" "	1957	Same	7.5
Same	"Volkhov-2"	Leningrad Oblast	1943	Ticks, Ixodes persulcatus	9.5
Same	256	Belorussia	1940	Ticks, Ixodes ricinus	8.0
Same	"DM"	Czechoslovakia	1954	Ticks, Dermacentor marginatus	7.6
Diphasic meningo-encephalitis	"Absettarov"	Karelian Isthmus	1951	Blood of a patient	7.5
Same	K-54	Moscow Oblast	1953	Goat milk	7.5
Same	"P-n"	Moscow	1957	Blood of a patient	8.3
Louping ill	I-40	Received from the USA	1944*	Not shown	9.3
Same	"MO"	Received from England	1947*	Brain of an ill sheep	6.5

* The date represents the time of receipt of the strain.

Table 2

The pattern of virus accumulation in the cultures of monkey-heart cells according to the days after inoculation (in lg LD_{50})

Virus	Strain	Days after inoculation									
		2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th
Tick-borne encephalitis	"Sof'in"	5.7	5.5	-	5.7			4.7			4.4
Diphasic meningo-encephalitis	"Abset-tarov"	6.3		6.7		6.6		4.3		2.3	
	"P-n"	6.6	6.5		4.7			3.5			3.0
Louping ill	I-40	7.8		7.7		7.1		4.7		3.3	
	"MO"	5.3		5.6		4.0		3.3		2.3	

Table 3

Comparative titration of the viruses in the cultures of monkey-heart cells and on white mice.

	Strains				
	"Sof'in" (5)*	"DN" (6)	"Abset-tarov" (6)	"P-n" (5)	"MO" (6)
The tissue cultures in lg $TTsPD_{50}$	5.3	5.3	5.5	6.0	5.0
The white mice in lg LD_{50}	5.5	6.0	6.0	6.5	5.7

* the figure in parentheses indicates the number of the cultural passage.